Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase I

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Luteolin, a naturally occurring flavonoid, is abundant in our daily dietary intake. It exhibits a wide spectrum of pharmacological properties, but little is known about its biochemical targets other than the fact that it induces topoisomerase II-mediated apoptosis. In the present study, we show that luteolin completely inhibits the catalytic activity of eukaryotic DNA topoisomerase I at a concentration of 40 μM, with an IC_{50} of 5 μM. Preincubation of enzyme with luteolin before adding a DNA substrate increases the inhibition of the catalytic activity ($IC_{50} = 0.66$ μM). Treatment of DNA with luteolin before addition of topoisomerase I reduces this inhibitory effect. Subsequent fluorescence tests show that luteolin not only interacts directly with the enzyme but also with the substrate DNA, and intercalates at a very high concentration (> 250 μM) without binding to the minor groove. Direct interaction between luteolin and DNA does not affect the assembly of the enzyme–DNA complex, as evident from the electrophoretic mobility-shift assays. Here we show that the inhibition of topoisomerase I by luteolin is due to the stabilization of topoisomerase-I DNA-cleavable complexes. Hence, luteolin is similar to camptothecin, a class I inhibitor, with respect to its ability to form the topoisomerase I-mediated 'cleavable complex'. But, unlike camptothecin, luteolin interacts with both free enzyme and substrate DNA. The inhibitory effect of luteolin is translated into concanavalin A-stimulated mouse splenocytes, with the compound inducing SDS–K^+-precipitable DNA–topoisomerase complexes. This is the first report on luteolin as an inhibitor of the catalytic activity of topoisomerase I, and our results further support its therapeutic potential as a lead anti-cancer compound that poisons topoisomerases.

Key words: cleavable complex, DNA intercalator, inhibitor, topoisomerase poison.

INTRODUCTION

DNA topoisomerases are key enzymes that catalyse the interconversion of topological isomers of DNA molecules. They act by the sequential breakage and reunion of either one strand (topoisomerase I) or both the strands of DNA (topoisomerase II) and are involved in many vital cellular processes, e.g. replication, transcription, recombination, integration and chromosomal segregation [1]. In recent years, the pharmacological inhibition of these enzymes has gained special interest, since topoisomerase inhibitors have emerged as anti-cancer [2], anti-microbial [3] and anti-parasitic agents [4].

Topoisomerase inhibition can be achieved by two distinct mechanisms, and the inhibitors are accordingly divided into two classes: class I and II inhibitors. Class I inhibitors stabilize the enzyme–DNA covalent complex and block the subsequent rejoining of the DNA break. Class II inhibitors, also referred to as catalytic inhibitors, prevent the enzyme–DNA binding by interacting with either topoisomerase [5,6] or DNA [7].

Topoisomerase II is the target of various anti-tumour agents, e.g. amsacrine, etoposide, teniposide and doxorubicin, which stabilize the 'cleavable complex' between enzyme and DNA [2]. Catalytic inhibitors that display high activity against topoisomerase II have been identified previously, e.g. merbarone [6] and aclarubicin [7]. In contrast, DNA topoisomerase I inhibitors are very rare, the most widely studied and characterized inhibitor being camptothecin, a topoisomerase I poison [8]. A few other topoisomerase I inhibitors have also been reported, e.g. β-lapachone [9], boswellic acid [10] and diospyrin [4].

Flavonoids are ubiquitously occurring and widely consumed secondary metabolites of plants and have profound pharmacological properties [11,12]. They are reported to have anti-viral [13], anti-parasitic [14] and anti-cancer [15] activities. Luteolin (3′,4′,5,7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetables and has contributed to the antioxidant activity of artichoke leaf extract on reactive oxygen species in human leucocytes [16]. Luteolin is also reported to have anti-inflammatory properties and mediates its action by inhibiting of nitric oxide production [17]. Luteolin has anti-allergic properties [18], and a recent report [19] establishes luteolin as a potent inhibitor of human mast cell activation through the inhibition of protein kinase C activation and Ca^{2+} influx. Luteolin exerts growth inhibitory effects on NK/Ly ascites-tumour-cell cultures *in vivo* [20]. The growth and metabolism of human leukaemic CEM-C1 and CEM-C7 cell lines are also inhibited by luteolin [21]. The flavonoids luteolin and quercetin are also reported to arrest cell cycle in the G_1 phase of human melanoma cells [22]. We have previously established that luteolin and quercetin inhibit DNA topoisomerase II of *Leishmania* and that they can induce cell-cycle arrest leading to

Abbreviations used: CT, calf thymus; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; *m*-AMSA, 4′-(acridinylaminol)-*N*-(methanesulphonyl)-*m*-anisidine.

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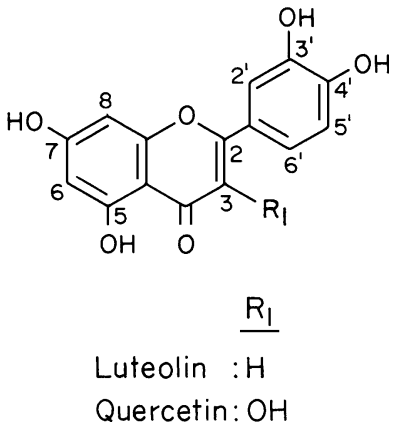

Figure 1 Structure of luteolin and quercetin

apoptosis of *Leishmania donovani* promastigotes [14]. Quercetin, an intercalator of DNA and responsible for site-specific cleavage in mammalian cells [23], is also reported to inhibit DNA topoisomerase I by preventing the re-ligation reaction [5]. But, unlike the case of quercetin, no reports are available as yet on the effect of luteolin on eukaryotic DNA topoisomerase I.

In the present study, we demonstrate that luteolin (Figure 1) is a potent inhibitor of eukaryotic DNA topoisomerase I. The binding of luteolin with the enzyme is dose-dependent, reversible and does not inhibit the subsequent enzyme–DNA duplex formation. At 250 μM, luteolin intercalates with DNA but does not bind to the minor groove. It induces stabilization of the cleavable complex both *in vitro* and within the cells. Our experiments show that inhibition of the catalytic activity of eukaryotic topoisomerase I by luteolin is mainly due to the stabilization of the cleavable complex, and intercalation does not play any significant role.

MATERIALS AND METHODS

Isolation of flavonoids

Luteolin was isolated from the leaves of *Vitex negundo* Linn by the method described previously [24]. The purity of the compound was attained by repeated crystallization from chloroform/methanol (19:1, v/v; m.p., 328–333 °C) and confirmed based on the following: by the appearance of a single spot in TLC [adsorbent silica gel G, TLC grade; Merck, Mumbai, India; developer, chloroform/methanol (49:1, v/v)], by the recording of a single molecular ion peak in MS, and by HPLC [Delta PAK, 5 μm, C-18, 300 Å, 3.9 mm × 150 mm; solvent: acetonitrile/water (4:1, v/v)]. The structure of the compound was ascertained by spectroscopic analysis and by superimposable IR spectra and undepressed mixed melting point with authentic samples.

Enzymes, DNA and chemicals

DNA topoisomerase I was purified from rat liver nuclei using the procedure described by Champoux and McConaughy [25]. Supercoiled pGEM4Z and pHOT1 DNA were purchased from Promega (Madison, WI, U.S.A.) and Topogen (OH, U.S.A.) respectively. pHOT1 DNA contains the high-affinity topoisomerase I cleavage site [26], which is derived from the *Tetrahymena* ribosomal gene repeat (hexadecameric sequence). Oligonucleotide 1 (5′-AAAAAGACTTAGAAAAATTTTTAAA-3′), oligonucleotide 2 (5′-TTTAAAAATTTTTCTAAGTCTTTTT-3′) and RPMI 1640 medium were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). These oligonucleotides, after annealing to double-stranded DNA, represent the high-affinity binding site for eukaryotic topoisomerase I [1]. [*methyl*-^{3}H]Thymidine (specific radioactivity, 86.0 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Concanavalin A was purchased from Roche Molecular Biochemicals (Germany). Electrophoresis-grade agarose, DMSO, 4′-(acridinylaminol)-*N*-(methanesulphonyl)-*m*-anisidine (*m*-AMSA, amsacrine), etoposide, quercetin and all other chemicals of high purity were purchased from Sigma.

Drug solutions

Luteolin, quercetin, *m*-AMSA, betulinic acid and etoposide were all dissolved in 100 % DMSO at 20 mM concentration and kept in aliquots at −20 °C. The final concentration of DMSO was kept at 4 and 0.5 % for the *in vitro* and cellular experiments respectively.

DNA relaxation assay

DNA topoisomerase I was assayed by measuring the decreased mobility of the relaxed isomers of supercoiled pGEM4Z DNA in an agarose gel following treatment with rat liver topoisomerase I. The standard topoisomerase assay mixture (25 μl) contained 50 mM Tris/HCl (pH 7.5), 5 % (v/v) glycerol, 50 mM KCl, 0.5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 30 μg/ml BSA, 0.5 μg of pGEM4Z DNA and 2 units of enzyme (1 unit is defined as the amount of enzyme required to convert 50 % of 0.5 μg of supercoiled DNA substrate into the relaxed form under standard assay conditions). Reactions were performed at 37 °C for 30 min, and then terminated by adding 10 mM EDTA, 0.5 % SDS, 0.25 μg/ml Bromophenol Blue and 15 % (v/v) glycerol. The samples were electrophoresed in a horizontal 1 % agarose gel in TAE buffer (40 mM Tris/acetate, 2 mM EDTA, pH 8) at 1.5 V/cm for 14–16 h at room temperature. The gels were stained with ethidium bromide (0.5 μg/ml), destained in water and photographed under UV illumination. Percentage of relaxation was measured by microdensitometry of negative photographs of supercoiled monomer DNA band fluorescence after ethidium bromide staining with a microdensitometer (Bromma 2202 Ultrascan, LKB), and the area under the peak was calculated.

Fluorescence spectroscopy

Fluorescence spectra were measured on a Hitachi-4500 fluorescence spectrophotometer. All fluorescence measurements were performed at an excitation wavelength of 380 nm and emission range of 450–650 nm. Excitation and emission slit widths were 5 nm. Background emission (< 2 %) was corrected by the subtraction of spectra of blank buffer and enzyme buffer from DNA + buffer samples and luteolin + enzyme samples respectively. The absorbance of samples at the excitation wavelength was less than 0.05. Spectral titrations were performed with 4×10^{-5} M luteolin at 25 °C in fluorescence buffer (20 mM Tris/HCl, pH 7.5, and 10 mM $MgCl_2$). DNA or topoisomerase I was added in increasing concentrations as indicated in the legends to Figures.

Luteolin–DNA intercalation

Intercalation between luteolin and DNA was assessed by two independent techniques. First, the ability of the drug to intercalate with pGEM4Z DNA was determined by the method described previously [27]. Assays were performed in the presence or absence of luteolin, *m*-AMSA, etoposide or quercetin in 40 μl of 50 mM

Tris/HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 30 μg/ml BSA and 0.5 μg of relaxed pGEM4Z DNA. Relaxed DNA was prepared by treatment of the supercoiled plasmid with excess topoisomerase I, followed by proteinase K digestion at 37 °C, phenol–chloroform extraction and ethanol precipitation. After incubation at 37 °C for 30 min, reactions were terminated by the addition of prewarmed stopping solution [5% SDS, 15% (w/v) Ficoll and 0.25% Bromophenol Blue] and electrophoresed on 1% agarose gel in TAE buffer as described above. The DNA bands were stained with 0.5 μg/ml of ethidium bromide, visualized by UV light and quantified as described above.

Secondly, an ethidium-displacement fluorescence assay [6] was employed to determine whether luteolin binds in the minor groove of DNA. Fluorescence emission spectra (λ_{max} = 590 nm, excitation wavelength 510 nm) were obtained at 25 °C. The assays contained 1 μM ethidium bromide, 0–300 μM luteolin, quercetin, etoposide or *m*-AMSA and 5 nM calf thymus (CT) DNA in 2 ml of fluorescence buffer.

Analysis of topoisomerase I–DNA interaction by electrophoretic mobility-shift assay (EMSA)

The substrate DNA used in the present study was a 25-mer duplex of oligonucleotide 1 and 2, containing a topoisomerase I-binding motif. The duplex was prepared by annealing the two oligonucleotides as described previously [28]. Annealed 25-mer duplex was incubated with 25 and 50 units of rat liver topoisomerase I at 8 °C for 15 min in the presence or absence of luteolin in 50 μl of binding buffer [10 mM Tris/HCl (pH 7.5), 3 mM $CaCl_2$, 50 mM NaCl, 0.1 M sucrose and 5% glycerol] [9]. After incubation, the reaction mixtures were electrophoresed in 7% non-denaturing polyacrylamide gel at 4 °C in 0.167 × TBE buffer (45 mM Tris/borate/1 mM EDTA) and DNA bands were stained with ethidium bromide as above.

DNA cleavage assay

Reaction mixtures (50 μl) containing 50 mM Tris/HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 mM DTT, 0.5 mM EDTA, 30 μg/ml BSA, 20 μg/ml pHOT1 DNA, 40 units of rat liver topoisomerase I and drugs were incubated at 37 °C for 30 min. The reactions were terminated by adding 1% SDS and 150 μg/ml proteinase K and further incubated for 1 h at 37 °C. DNA samples were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Measurement of DNA synthesis and SDS–K^+-precipitable protein–DNA complex in concanavalin A-stimulated mouse splenocytes

Mouse splenocytes were stimulated with concanavalin A (3 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum and 0.05 mM 2-mercaptoethanol as described previously [29]. After 72 h, stimulated T-cells were distributed in 96-well plates (10^6 cells/well) and labelled by adding [*methyl*-^{3}H]thymidine to a final concentration of 1 μCi/well. Cells (200 μl) in each well were then incubated for 3–12 h at 37 °C in a CO_2 incubator in the presence or absence of different concentrations of luteolin. Following incubation, cells were lysed with 10% trichloroacetic acid and harvested in a Skatron Combi cell harvester. The incorporation of radioactivity in acid-precipitable DNA was measured in a Wallac 1409 liquid scintillation counter.

The *in vitro* formation of covalent topoisomerase I–DNA complex was quantified using the SDS–K^+-precipitation assay as described previously [9]. Mouse splenocytes were stimulated with concanavalin A for 72 h as described above. DNA in stimulated T-cells (2×10^7 cells/ml) was labelled by adding [*methyl*-^{3}H]thymidine into the medium to a final concentration of 5 μCi/ml for 18 h. Cells were pelleted and washed twice with phosphate-buffered saline (137 mM NaCl/2.6 mM KCl/8.0 mM Na_2HPO_4/1.4 mM KH_2PO_4), resuspended in complete medium and distributed into 96-well plates at 5×10^6 cells/ml; the final volume was 200 μl/well. After incubation at 37 °C for 2 h, cells were treated with various concentrations of camptothecin, luteolin and/or betulinic acid for 30 min. The microtitre plate was centrifuged in a Beckmann RT 6000 centrifuge at 2500 rev./min for 2 min at room temperature. After removing the medium from the wells, cells were lysed by the addition of 200 μl of a prewarmed (65 °C) lysis solution [1.25% SDS, 5 mM EDTA (pH 8), CT DNA (0.4 mg/ml)]. The lysates were transferred to 1.5 ml microfuge tubes containing 250 μl of 325 mM KCl. After vigorous vortex mixing, the samples were cooled in ice for 10 min and centrifuged. The pellets were resuspended in 500 μl of wash solution [10 mM Tris/HCl (pH 8), 100 mM KCl, 1 mM EDTA, CT DNA (0.1 mg/ml)] and warmed at 65 °C for 10 min with occasional shaking. The suspensions were cooled in ice for 10 min and re-centrifuged. The pellets were washed again before resuspending in 200 μl of water prewarmed at 65 °C. The suspensions were mixed with 4 ml of scintillation liquifluor (Spectrochem, Bombay, India) and the radioactivity was determined in the liquid scintillation counter.

RESULTS

Inhibition of topoisomerase I catalytic activity by luteolin

The inhibition of catalytic activity of purified rat liver topoisomerase I by luteolin was studied *in vitro* by DNA relaxation assay [4] in the absence of ATP (Figure 2A). When added together with DNA and enzyme, luteolin partially inhibited relaxation of supercoiled DNA at 1 μM concentration and more

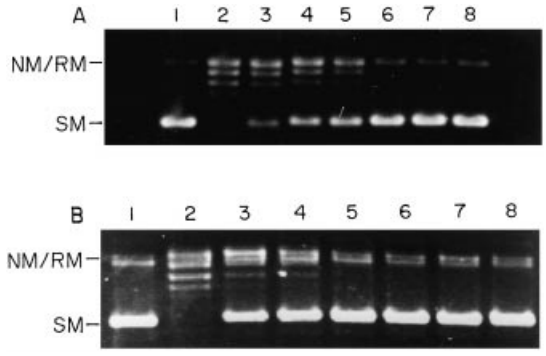

Figure 2 Inhibition of catalytic activity of rat liver topoisomerase I by luteolin

(**A**) Simultaneous addition of enzyme, luteolin and DNA. Lane 1, supercoiled pGEM4Z DNA; lane 2, DNA with 2 units of purified rat liver topoisomerase I and 4% DMSO; lanes 3–8, inhibition of catalytic activity in the presence of 1, 5, 10, 20, 40 and 80 μM luteolin. (**B**) Preincubation of enzyme with luteolin followed by addition of DNA. Lane 1, supercoiled GEM4Z DNA; lane 2, DNA was added after preincubation of 2 units of topoisomerase I with the reaction buffer containing 4% DMSO for 5 min at 37 °C; lanes 3–8, same as lane 2, but the enzyme was preincubated with 1, 5, 10, 20, 40 and 80 μM luteolin respectively. Samples were electrophoresed in 1% agarose gel as described in the Materials and methods section. Positions of supercoiled monomer (SM), nicked monomer (NM) and relaxed monomer (RM) are indicated.

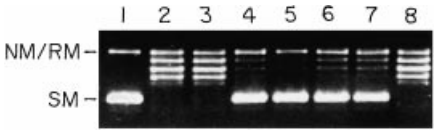

Figure 3 Luteolin–enzyme interaction is reversible

Preincubation and dilution with 1 μM luteolin. Lane 1, supercoiled pGEM4Z DNA alone; lane 2, 2 units of preincubated enzyme control; lanes 3–5, same as lane 2, but in the presence of 0.2, 0.5 and 1 μM luteolin respectively. Topoisomerase I (2 units) was preincubated with 5 μM luteolin for 5 min at 37 °C, then diluted 0-, 2- and 5-fold while maintaining or diluting the final drug concentrations to 1 μM (lane 6), 0.5 μM (lane 7) and 0.2 μM (lane 8) respectively. Reactions were performed at 37 °C for 15 min after addition of 0.5 μg of supercoiled pGEM4Z DNA.

effectively at higher concentrations (Figure 2A, lanes 3–8). Lane 2 shows the relaxation of supercoiled pGEM4Z DNA (lane 1) by 2 units of topoisomerase I. The inhibitory effect of luteolin was significantly increased by preincubating the compound with topoisomerase I for 5 min at 37 °C before the addition of DNA substrate (Figure 2B). Lane 3 shows that the compound exerts 80% inhibition at 1 μM and 95% inhibition at 5 μM concentration (lane 4). However, at this 5 μM concentration, only 50% inhibition was attained when enzyme, DNA and luteolin were added together.

Luteolin by itself did not relax DNA. To test this, supercoiled pGEM4Z was incubated with luteolin at concentrations up to 200 μM without the addition of topoisomerase I. No relaxation and no apparent DNA conformational changes took place because of the unwinding of DNA (results not shown).

Luteolin is acting reversibly against the enzyme

Since luteolin inhibits the catalytic activity of rat liver topoisomerase I, the next and most important issue is to understand the mechanism of inhibition. It is not clear whether luteolin is acting reversibly or irreversibly against the enzyme. This critical matter has been sorted out by the dilution experiment. Topoisomerase I was preincubated with 1 μM luteolin (Figure 3, lane 5), a concentration at which 80% inhibition is achieved, before the addition of DNA. The reaction mixtures were subsequently diluted 2- and 5-fold so that the final drug concentration becomes 0.5 and 0.2 μM respectively. Drug-control reactions, i.e. inhibition study with 0.2 and 0.5 μM luteolin, showed the expected pattern of inhibition (lanes 3 and 4). Dilution from 1 to 0.2 μM resulted in a complete relief of inhibition (lane 8). Lane 2 serves as the enzyme control. This relief of inhibition on dilution suggests that luteolin is acting reversibly against topoisomerase I.

Fluorescence spectroscopy was also used to check directly the binding of luteolin to topoisomerase I (results not shown). Addition of enzyme to luteolin led to an increase in the fluorescence intensity of luteolin without changing the fluorescence maxima ($\lambda_{max} = 525$ nm). However, from this change in fluorescence it is not possible to quantify this interaction, since the amount of enzyme available is small, and equimolar concentrations of enzyme, as compared with the drug, cannot be achieved.

Luteolin–DNA preincubation reduces the inhibitory effect

Having determined that luteolin binds reversibly to topoisomerase I, the effects of luteolin, if any, on supercoiled

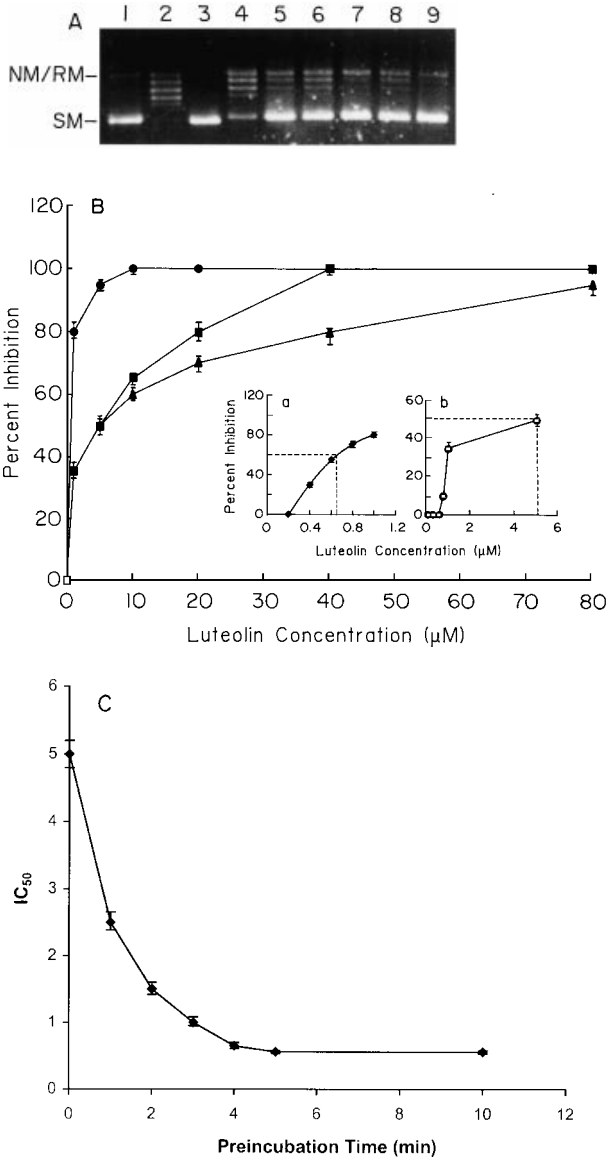

Figure 4 Depletion of luteolin inhibitory effect upon preincubation with DNA

(**A**) Luteolin–DNA interaction depletes inhibition of topoisomerase I catalytic activity. The experimental method used is described in the Materials and methods section. Lane 2, relaxation of supercoiled pGEM4Z DNA (lane 1) in the presence of 2 units of topoisomerase I; lane 3, inhibition of enzyme activity by the simultaneous addition of 40 μM luteolin; lane 4, preincubation of pGEM4Z DNA with 1 μM luteolin for 5 min at 37 °C, followed by the addition of 2 units of topoisomerase I and further incubation at 37 °C for 30 min. Lanes 5–9, same as lane 4, but with 5, 10, 20, 40 and 80 μM luteolin respectively. Positions of supercoiled monomer (SM), nicked monomer (NM) and relaxed monomer (RM) are indicated. (**B**) Densitometric scanning of topoisomerase I inhibition by luteolin. (▲), Preincubation of DNA with luteolin followed by addition of 2 units of topoisomerase I; (■), simultaneous addition of enzyme, DNA and luteolin; (●), preincubation of luteolin with topoisomerase I followed by the addition of DNA. Inset: IC_{50} determination of (a) luteolin–enzyme preincubation (◆); (b) luteolin–enzyme simultaneous addition (●) and luteolin–DNA preincubation (□) (symbols are superimposed). All experiments were performed three times and representative data from one set of these experiments are expressed as means ± S.D. Variation among different sets of experiments was less than 5%. (**C**) Variation of IC_{50} with preincubation time (◆). The IC_{50} values were calculated for each type of preincubation from a plot of inhibition percentage versus luteolin concentration. All experiments were performed three times and representative data from one set of these experiments are expressed as means ± S.D. Variation among different sets of experiments was less than 5%.

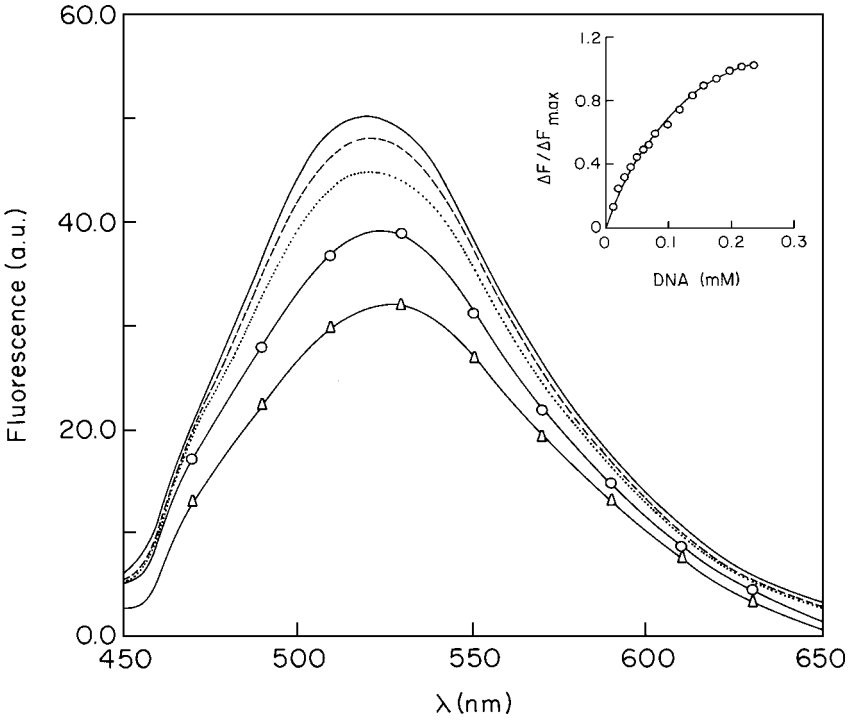

Figure 5 Fluorescence study of luteolin–DNA interaction

A change in the fluorescence emission spectrum of luteolin (4×10^{-5} M) (△) was observed on addition of CT DNA at concentrations of 5×10^{-5} M (○), 1.57×10^{-4} M (· · ·), 2.72×10^{-4} M (----) and 3.47×10^{-4} M (—). All measurements were made after preincubating luteolin with or without DNA at 37 °C for 5 min. Inset: plot of $\Delta F/\Delta F_{max}$ versus increasing concentration of CT DNA.

substrate DNA were next studied (Figure 4A). Experiments were performed by preincubating different concentrations of luteolin with pGEM4Z DNA at 37 °C for 5 min before the addition of enzyme and further incubated for 30 min at 37 °C. Lane 3 shows that 100 % inhibition of relaxation was achieved by luteolin at 40 μM when added simultaneously with enzyme and DNA. However, when 40 μM luteolin was preincubated with DNA before addition of enzyme, the inhibition observed was 80 % (lane 8), and even 80 μM luteolin did not lead to 100 % inhibition of the enzyme activity (lane 9). The reduction of enzyme inhibition by luteolin upon preincubation with DNA and the marked enhancement of inhibition upon preincubation of enzyme with luteolin are represented by densitometric analysis of the agarose gels (Figure 4B). The experiments were performed three times, and representative data from one set of these experiments are presented as means ± S.D. Variation among different sets of experiments was less than 5 %.

The IC_{50} values for enzyme preincubation, simultaneous addition and DNA preincubation were calculated from the inset to Figure 4(B). Comparison of IC_{50} under enzyme preincubation (0.66 μM) and simultaneous addition (5 μM) again highlights the inhibitory potency of luteolin upon preincubation. However, DNA–luteolin preincubation does not affect the IC_{50} (5 μM), despite reduction of the inhibitory effect at higher concentrations. IC_{50} values were also determined under different preincubation conditions. Luteolin was preincubated with enzyme for 1–5 min and 10 min before the addition of substrate DNA and further incubated at the same temperature for 30 min. Results showed that IC_{50} decreased with increasing preincubation time and it was minimal for 5 min preincubation. Further preincubations had no effect on IC_{50} (Figure 4C).

Luteolin–DNA interaction as studied by fluorescence titration

The reduction of the inhibitory effect of luteolin after preincubation with DNA led us to investigate the ability of the compound to interact with DNA by fluorescence titration with CT DNA. Free luteolin has fluorescence emission maximum at 525 nm. Addition of CT DNA causes a slight shift of the maximum peak from 525 to 519 nm with a concomitant increase in fluorescence intensity (Figure 5). A progressive change in the fluorescence spectra of luteolin on addition of different concentrations of DNA indicated an association between them. A plot of $\Delta F/\Delta F_{max}$ versus concentration of DNA was found to be hyperbolic, indicating the formation of a saturable complex (inset to Figure 5). The dissociation constant K_D calculated from these values using Scatchard analysis [30] is 4.6×10^{-5} M.

Luteolin intercalates with DNA at higher concentrations

Our next aim was to elucidate the mode of interaction between luteolin and DNA and extrapolate its effect, if any, on topoisomerase I inhibition. Many chemical compounds are reported to alter the gross structure of DNA either by intercalation and/or by minor-groove binding [31,32], which in turn can affect the catalytic activity of topoisomerase I. Since luteolin interacts with the substrate DNA, it is possible that luteolin inhibits topoisomerase I by one of these two mechanisms. Two approaches were followed to answer this question.

First, the ability of luteolin to intercalate with DNA was determined by a topoisomerase I-catalysed unwinding assay (Figure 6A), which is based on the ability of intercalating compounds to unwind the DNA duplex and thereby change the DNA twist [33]. The DNA substrate was purified topological

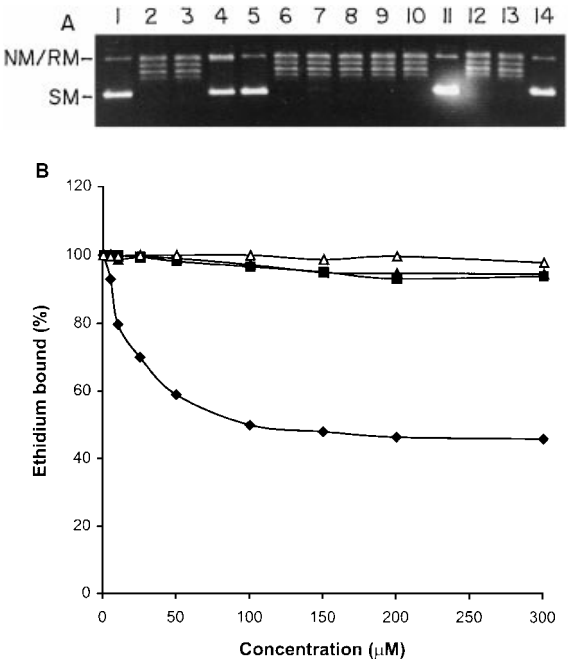

Figure 6 Luteolin intercalates with DNA without binding to the minor groove

(**A**) Luteolin–DNA intercalation as studied by agarose-gel electrophoresis. Lane 2, relaxed pGEM4Z DNA generated by treatment of supercoiled pGEM4Z (lane 1) with excess topoisomerase I, followed by phenol–chloroform extraction and ethanol precipitation; lanes 3–5, 10, 50 and 250 μM *m*-AMSA respectively; lanes 6–8, 10, 50 and 250 μM etoposide respectively; lanes 9–11, 10, 50 and 250 μM quercetin respectively; lanes 12–14, 10, 50 and 250 μM luteolin respectively. (**B**) Luteolin does not displace ethidium bromide from the minor groove of DNA. The ability of luteolin to interact with the minor groove of DNA was determined by a fluorescence-based ethidium bromide displacement assay. Samples contained 1 μM ethidium bromide and 5 nM CT DNA. Increasing concentrations of luteolin (■), quercetin (▲), *m*-AMSA (◆) and etoposide (△) were added, and ethidium fluorescence at 590 nm (λ_{max}) was monitored (510 nm excitation wavelength).

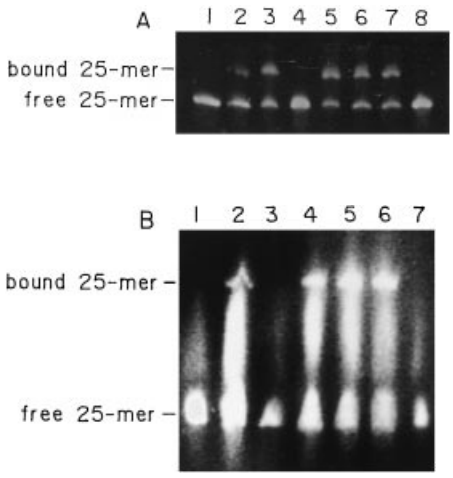

Figure 7 Luteolin does not prevent binding of topoisomerase I to substrate DNA

(**A**) EMSA with 10–50 μM luteolin. The experimental method used is described in the Materials and methods section. Lane 1, 25-mer duplex oligonucleotide in binding buffer alone; lanes 2 and 3, same as lane 1, but incubated with 25 and 50 units respectively of topoisomerase I at 8 °C for 15 min; lane 4, same as lane 3, followed by treatment with SDS–proteinase K for 1 h at 37 °C; lane 5, 10 μM luteolin preincubated with 50 units of enzyme for 5 min at 37 °C before the addition of substrate DNA and further incubated at 8 °C for 15 min; lanes 6 and 7, same as lane 5, but with 50 and 100 μM luteolin respectively; lane 8, incubation of 25-mer duplex oligonucleotide with 100 μM luteolin at 37 °C for 5 min. (**B**) EMSA with 300 μM luteolin. Lane 1, 25-mer duplex oligonucleotide in binding buffer alone; lane 2, 25-mer plus 50 units of topoisomerase I; lane 3, same as lane 2 followed by treatment with SDS–proteinase K for 1 h at 37 °C; lane 4, 300 μM luteolin preincubated with 50 units of enzyme for 5 min at 37 °C before the addition of substrate DNA and further incubated at 8 °C for 15 min; lane 5, 300 μM luteolin incubated simultaneously with 50 units of topoisomerase I and 25-mer duplex oligomer at 8 °C for 15 min; lane 6, 300 μM luteolin preincubated with 25-mer duplex oligomer at 37 °C for 30 min before the addition of 50 units of topoisomerase I and further incubated at 8 °C for 15 min; lane 7, incubation of 25-mer duplex oligonucleotide with 100 μM luteolin at 37 °C for 5 min.

isomers of supercoiled pGEM4Z DNA as described in the Materials and methods section. In the presence of a strongly intercalative drug such as *m*-AMSA, a net negative supercoiling of relaxed substrate DNA was induced (lanes 4 and 5). Conversely, no unwinding was observed with the non-intercalative drug etoposide (lanes 6–8). Luteolin, at a concentration of 250 μM, induced a net negative supercoiling (lane 14) similar to *m*-AMSA (lanes 4 and 5) and quercetin (lane 11). These findings strongly suggest that at high concentration (250 μM), luteolin intercalates with DNA and is similar to the related flavonoid, quercetin [23].

In the second experiment, the ability of luteolin to displace ethidium bromide from DNA was determined by fluorescence emission assay [6]. Free ethidium bromide has a significantly weaker fluorescence compared with the DNA bound form; thus displacement of ethidium from DNA can be monitored by a decrease in the fluorescence signal [34]. Moreover, since ethidium bromide intercalates with DNA through interactions in the minor grove, the compounds that either intercalate and/or bind in the minor groove of DNA are capable of responding to this assay. As shown in Figure 6(B), the intercalative drug *m*-AMSA readily dislodged the bound fluorophore, whereas the non-intercalative drug etoposide was unable to do so. Quercetin, a weak intercalator, causes 6% displacement at a concentration of 300 μM. Similar displacement took place with luteolin at that concentration. Taken together, these results indicate that though luteolin intercalates with DNA at high concentrations, it does not bind to the minor groove of DNA. Moreover, this very high concentration (> 250 μM) compared with the very low IC_{50} of topoisomerase I inhibition (0.66 μM) suggests that the mode of inhibition is something different from luteolin–DNA intercalation.

Luteolin does not prevent binding of topoisomerase I to the substrate DNA

The first step of a topoisomerase-catalysed reaction is the binding of the enzyme to the substrate DNA. To elucidate the mode of inhibition of luteolin, we investigated the ability of luteolin to interfere with the enzyme–DNA binding by an EMSA (Figure 7A). Excess topoisomerase I was added to ensure a stronger shift. Incubation of 25-mer double-stranded oligonucleotide (lane 1) with 25 units of topoisomerase I formed a protein–DNA complex, which is visible as a retarded band (lane 2). With 50 units of the enzyme, intensity of the retarded band also increases (lane 3). However, treatment of the enzyme–DNA complex with SDS–proteinase K released the DNA (lane 4). Luteolin, up to a concentration of 100 μM, neither inhibited the formation of enzyme–DNA complex (lanes 5–7) nor changed the electrophoretic mobility of the 25-mer duplex oligonucleotide (lane 8).

However, at 250 μM concentration, luteolin intercalates with DNA, which might prevent the binary complex formation

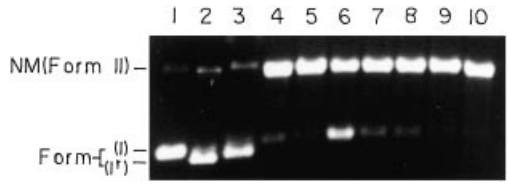

Figure 8 Luteolin stabilizes topoisomerase I-mediated DNA cleavage

Cleavage reaction and electrophoresis in agarose gel were performed as described in the Materials and methods section. Lane 1, 1 μg of supercoiled pHOT1 DNA; lane 2, with 40 units of rat liver topoisomerase I; lane 3, same as lane 2, but with SDS–proteinase K treatment; lanes 4 and 5, same as lane 3, but in the presence of 10 and 50 μM camptothecin respectively; lanes 6–10, same as lane 3, but in the presence of 1, 5, 10, 20 and 50 μM luteolin. Positions of supercoiled substrate (form I), relaxed closed DNA molecules (form I^r) and nicked monomer (form II) are indicated.

between topoisomerase I and DNA. To determine this, EMSA was performed with 300 μM luteolin (Figure 7B). Topoisomerase I (50 units) was added to 25-mer double-stranded oligonucleotide (lane 1) and the protein–DNA covalent complex is visible as the retarded band (lane 2) that disappears on SDS–proteinase K treatment (lane 3). Neither the preincubation of enzyme with 300 μM luteolin nor the simultaneous addition of 300 μM luteolin and enzyme prevented the formation of this binary complex (lanes 4 and 5). Interestingly, preincubation of oligomer with 300 μM luteolin at 37 °C for 30 min followed by enzyme addition also does not inhibit the enzyme–DNA binding (lane 6). Mobility of the 25-mer remains unchanged when preincubated with 300 μM luteolin (lane 7). The same experiment was repeated with supercoiled plasmid DNA and similar results were obtained (results not shown). Therefore from this experiment it is evident that luteolin does not inhibit the binding of topoisomerase I to DNA when it intercalates with DNA.

Luteolin induces topoisomerase I-mediated DNA cleavage

The second step of a topoisomerase I-catalysed reaction is the introduction of a single-stranded nick in the phosphodiester bond of the DNA, through which an intact strand is allowed to pass. During this process, a covalent bond is formed between the 3′-phosphoryl group of the DNA backbone and the tyrosine residue at the active site of topoisomerase I. This covalent complex is the putative reaction intermediate and is popularly known as the 'cleavable complex'. It can be detected by the addition of strong protein denaturants like NaOH or SDS. Camptothecin, a widely studied topoisomerase I inhibitor, has been shown to stabilize the cleavable complex [8]. In the present study we investigated the ability of luteolin to stabilize the cleavable complex formation between topoisomerase I and pHOT1 DNA (Figure 8, lane 1). Closed circular DNA (form I) was converted into nicked circular DNA (form II) in the presence of 40 units of topoisomerase I and 50 μM camptothecin (lane 5). With 1 μM luteolin, 50 % of form I DNA was converted into form II (lane 6). With increasing concentrations of luteolin, the amount of form II DNA increased (lanes 7–10), and complete cleavage was induced by 20 μM luteolin. The background cleavage, i.e. the formation of form II DNA in the presence of topoisomerase I only, is shown in lane 2. Lane 3 shows the formation of nicked product when the covalent complex was trapped with SDS and proteinase K. Ethidium bromide at a final concentration of 0.5 μg/ml was included in the gel to resolve the more slowly migrating nicked product (form II) from the relaxed molecules (form I^r) as described previously [4]. This result shows that

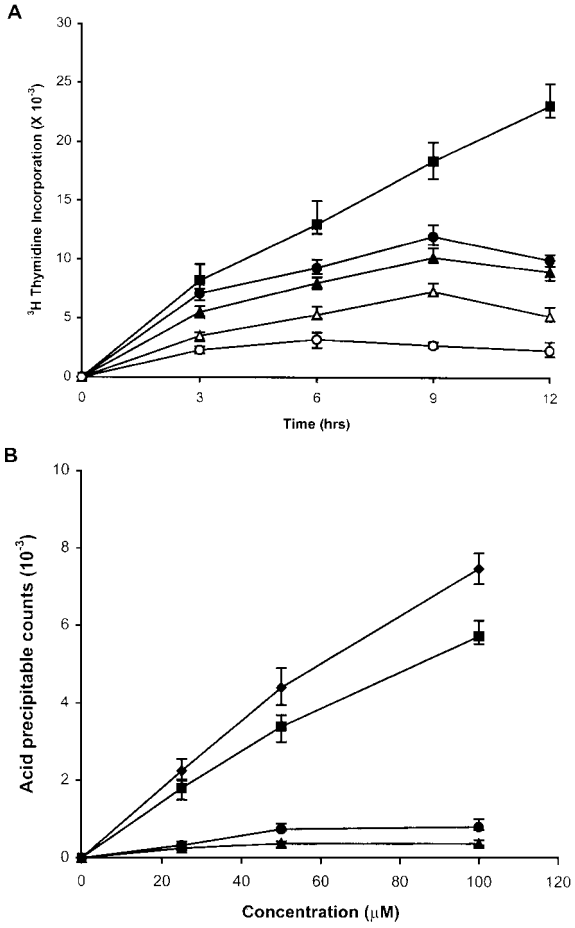

Figure 9 Luteolin inhibits DNA synthesis of concanavalin A-stimulated mouse splenocytes and induces the formation of SDS–K^+-precipitable protein–DNA complex within cells

(**A**) Incorporation of [^{3}H]thymidine (5 μCi/ml) into DNA of concanavalin A-activated mouse splenocytes were monitored following the addition of [^{3}H]thymidine and 0.5% DMSO (■), and the addition of luteolin at 12.5 μM (●), 25 μM (▲), 50 μM (△) or 100 μM (○) to the cell cultures (5×10^6 cells/ml) at zero time. Aliquots of 50 μl each were withdrawn from the cultures at the indicated time intervals and processed to determine the incorporation of label into acid-precipitable DNA. The experiments were performed three times and representative data from one set of these experiments are presented as means ± S.D. A 5% variation was seen among the different sets of experiments. (**B**) Stimulated mouse splenocytes were labelled with [^{3}H]thymidine for 18 h and then treated with different concentrations of luteolin, camptothecin or betulinic acid. The total acid-precipitable counts per assay were determined to be 2.6×10^4 c.p.m. Parts of the labelled cells were treated with betulinic acid (100 μM) for 10 min before the addition of different concentrations of luteolin. (■), camptothecin; (◆), luteolin; (▲), betulinic acid; (●), betulinic acid plus luteolin. SDS–K^+-precipitable complex was measured as described in the Materials and methods section. The experiment was performed three times and representative data from one set of these experiments are presented as means ± S.D. A 7% variation was seen among the different sets of experiments.

luteolin does not inhibit step II of a topoisomerase I-catalysed reaction. Instead, it stabilizes the cleavable complex and acts as a topoisomerase poison.

Effect of luteolin on DNA synthesis of concanavalin A-stimulated mouse splenocytes

The effect of luteolin on DNA synthesis was studied by the incorporation of [^{3}H]thymidine into stimulated mouse splenocyte DNA in the presence and absence of different concentrations of luteolin (Figure 9A). Within 9 h of addition of the compound,

12.5 and 50 μM luteolin decreased [^{3}H]thymidine incorporation to 65 and 40 % of the control respectively. After 12 h, the incorporation decreased to 43 % with 12.5 μM luteolin and to 22.5 % with 50 μM luteolin. At 100 μM concentration of luteolin, incorporation decreased to 10 % after 12 h of incubation. Reduction of [^{3}H]thymidine incorporation with time at a particular concentration is indicative of DNA damage within splenocyte cells.

Luteolin induces the formation of SDS–K$^+$-precipitable complex within cells

The stabilization of cleavable complexes within cells was studied in stimulated mouse splenocytes by the SDS–K$^+$ precipitation assay. Experiments were performed in the presence and absence of luteolin. As shown in Figure 9(B), the SDS–K$^+$-precipitable complex in cells was greatly increased by luteolin, and the effect was more potent than that of camptothecin. On the contrary, betulinic acid, a pentacyclic triterpenoid and a catalytic inhibitor of both topoisomerase I and II, which is reported to antagonize camptothecin-mediated cleavage [10] (A. R. Chowdhury, S. Mandal, B. Mittra, S. Sharma, S. Mukhopadhyay, H. K. Majumder, unpublished work), did not induce the formation of SDS–K$^+$-precipitable complex. The antagonistic nature of betulinic acid is attributed to the fact that it binds very strongly with topoisomerases, unlike camptothecin, which does not bind, or luteolin, which interacts reversibly with the enzyme. To prove that the SDS–K$^+$-precipitable complex formation induced by luteolin is due to topoisomerase–DNA cross-links, the stimulated mouse splenocyte cells were pretreated with betulinic acid before incubation with luteolin. The SDS–K$^+$-precipitable complex formation induced by luteolin was clearly decreased when the cells were pretreated with betulinic acid (100 μM). These results establish that the SDS–K$^+$-precipitable complex is due to formation of covalent complexes between topoisomerases and DNA and not due to any other proteins cross-linked to DNA.

DISCUSSION

Our results show that luteolin is a potent inhibitor of DNA topoisomerase I, like camptothecin. But, unlike camptothecin, luteolin binds to both the enzyme and DNA individually. Interaction of luteolin with enzyme was indicated by fluorescence titration of luteolin with increasing concentrations of enzyme (results not shown). The enzyme–luteolin interaction was reversible, as studied by the dilution experiment. Interaction of luteolin with DNA was primarily indicated by the reduction of inhibitory effect when luteolin was preincubated with DNA. Direct measurement of this interaction was made by fluorescence spectroscopy. The K_D value of luteolin–DNA (4.6×10^{-5} M) is comparable with the topoisomerase II poisons *m*-AMSA (2.1×10^{-5} M) [35] and etoposide (5.8×10^{-5} M) [36], but it is much greater than that of ethidium bromide binding to DNA (4×10^{-7} M) [37], indicating less affinity. Since luteolin interacts with both the enzyme and the DNA, the compound is distributed between the enzyme and the substrate DNA during simultaneous reaction. When the enzyme is preincubated with luteolin, no such distribution takes place in the absence of DNA. As a result, IC_{50} decreases with increasing preincubation time; and after 5 min of preincubation, IC_{50} becomes 0.66 μM, which is very low compared with the IC_{50} of the simultaneous reaction (5 μM). Further preincubations do not alter the IC_{50}, indicating that the enzyme–luteolin interaction is complete within 5 min. However, there is no increase in IC_{50} (5 μM) when luteolin is preincubated with DNA, though reduction of the inhibitory effect is apparent at higher concentrations of the compound.

The luteolin–DNA interaction was further assessed by two independent DNA-binding assays, which showed luteolin to intercalate at a very high concentration (250 μM) without binding to the minor groove. By comparing such high intercalating concentrations with the concentration required for complete inhibition (5 μM), it becomes evident that the inhibition of relaxation reaction by luteolin is not due to its intercalation with DNA. Instead, it is more likely that the compound exerts its effect by a specific interaction with either the enzyme or the enzyme–DNA complex. Also, the slight reduction in luteolin inhibitory effect after preincubation with DNA (no change in IC_{50}) in contrast with the dramatic enhancement of inhibitory activity upon preincubation with enzyme and luteolin (IC_{50} changes from 5 to 0.66 μM) suggests that luteolin binding to DNA plays no significant role in drug action.

A topoisomerase reaction has three general mechanistic steps, i.e. (i) binding of the enzyme to the substrate DNA, (ii) strand breakage and subsequent strand passage through the break, leading to change in linking number, and (iii) strand re-ligation. The binding of luteolin and DNA topoisomerase I does not prevent the enzyme from its subsequent DNA binding, i.e. mechanistic step I of topoisomerase I-catalysed reaction, as evident from EMSA. Even at 300 μM, a concentration at which luteolin intercalates with the substrate DNA, enzyme–DNA binary complex formation is not inhibited. This confirms that luteolin does not prevent the access of topoisomerase I to DNA. Cleavage experiment shows that luteolin does not inhibit the formation of ternary complex, i.e. mechanistic step II of topoisomerase I-catalysed reaction. On the contrary, luteolin stabilizes the formation of cleavable complex, total cleavage being induced at 20 μM. Moreover, 300 μM luteolin still induces cleavage (results not shown), indicating that the mode of inhibition of luteolin remains unaltered even though it is intercalated with DNA. Thus luteolin inhibits topoisomerase I catalytic activity by acting as a classical topoisomerase I poison and it can be classified as class I inhibitor.

Subsequent cellular studies revealed that luteolin inhibited DNA synthesis and also induced stabilization of the protein–DNA cross-links. It is fair to comment that formation of topoisomerase–DNA cross-links is definitely one of the reasons for the inhibition of DNA synthesis by luteolin. Moreover, it again demonstrates that direct interaction between luteolin and DNA does not affect the assembly of enzyme–DNA complex and its subsequent stabilization by the compound in cellular systems.

At present luteolin is reported to have various pharmacological effects [16–19] and has evolved as an anti-tumour agent [20–22]. One more structurally related flavone is quercetin (3,3′,4′,5,7-pentahydroxyflavone), the most widely studied flavone to date. It is reported to inhibit the growth of human gastric cancer cells [15] and tumour cells [38], but comparative studies have established luteolin to be a more potent and specific therapeutic agent [14,38]. Recently [39], of the 27 citrus flavonoids examined for their anti-proliferative activities against several tumour and normal human cell lines, luteolin was found to be the most potent of all, ahead of quercetin. Therefore the therapeutic importance of luteolin as a lead compound for anti-cancer chemotherapy is greatly enhanced by the present study, which is the first study of the compound as eukaryotic topoisomerase I inhibitor.

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